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Genotype specific host resistance for *Phytophthora* in black pepper (*Piper nigrum* L.)

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ABSTRACT

Phytophthora foot rot is a devastating disease in black pepper which is caused by *P. capsici*. Using host resistance is a major component in the integrated pest management of this disease. To explore the host resistance pattern, we studied the genotype specific differential expression of important pathogenesis related gene β 1, 3 glucanase and 5 R gene loci from resistant and susceptible varieties of black pepper. The relative expression of β 1, 3 glucanase was up regulated in resistant variety and down regulated in susceptible variety. R gene loci Pn CNBS 5, CNBS 3 and Pn CNBS 2 showed up regulation in susceptible variety in the early hours of infection while resistant genotype recorded its down regulation till 72 h suggesting that the selective down expression of these loci may be attributed to the resistance in the resistant variety. These R genes may be the susceptibility genes (S-genes) in black pepper for *Phytophthora*. The high relative expression of the R gene locus Pn CNBS 4 and Pn CNBS 1 in resistant genotype at early hours shows their involvement in resistance towards *P. capsici*. The differential expression of the R gene loci shows the genotype specific functional significance for *Phytophthora* resistance.

1. Introduction

Black pepper popularly known as King of spices, native to India, is an export oriented important spice crop grown in tropical countries. The foot rot disease caused by an oomycete *Phytophthora capsici* contributes to major crop loss as it infects the vine both in nursery and in field [1]. The elucidation of host resistance in *P. nigrum* - *P. capsici* pathosystem is still in its infancy. The analysis on expression of [6,11] 1, 3 beta glucanase between resistant and susceptible varieties were done using western blotting study. Pathogenesis related proteins are the important candidate elements that are induced by pathogen infection and improve the genotype specific defensive capacity of plants. There are also several reports on over expressing pathogenesis related genes, resulting in enhanced tolerance to pathogen infection.

The majority of plant resistance proteins (R genes) belong to the NB-LRR super family, which directly or indirectly recognizes pathogen effectors. Host resistance is conferred by the presence of a functional allele of a resistance (R) locus that displays functional natural variation. The NB-LRR protein has N-terminus Toll and interleukin receptor (TIR) domain or a coiled coil (CC) domain that is involved in downstream signaling [13], the C terminal LRR region is involved in elicitor recognition specificity [2]. R genes mediating specific recognition of bacteria such as RPM1, RPS2, RPS4, RPS5and RRS1 or oomycetes such as RAC1, WRR4, RPP1, RPP2, RPP5, RPP7, RPP8, and RPP13 were functionally characterized in Arabidopsis [12]. RPP1, RPP2, RPP5, RPP7, RPP8 and RPP13 activate defense for downy mildew parasite, *Hyaloperonospora arabidopsidis exparasitica* (Hpa). In *A. thaliana*, most R loci and genes have been identified for Hpa elucidating its importance in At evolution [14].

The work regarding the R genes is nill in black pepper –*Phytophthora* pathosystem. Hence we attempt to study the differential expression of β 1, 3 glucanase and 5 R gene loci from resistant and susceptible genotypes of black pepper. The molecular information on defense related genes and its expression will provide a critical foundation for understanding the underlying mechanism of disease resistance, screening germplasm and improving disease resistance breeding programs in black pepper.

2. Materials & methods

The resistant (IISR Shakthi) and susceptible (Subhakara) varieties of black pepper were raised (3–4 leaf stage) in sterile potting mixture (1:1:1) (Soil: Sand: FYM) maintained in the green house

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were used in our study. A virulent isolate of *P. capsici* (05–06) obtained from National repository of *Phytophthora*, Indian Institute of Spices Research, Kozhikode was used to challenge inoculate the plants (Accession KC300238.1). The experiment was conducted in three replications each. Inoculum plugs of 3 mm size from 72 h old mycelial culture of *P. capsici* (05–06) cultured on carrot agar media were placed on the lower surface of the leaves and moist cotton strip was placed over the inoculum plug and was secured using cello tape. Plants with moist cotton on the lower surface of the leaves served as control.

Leaf samples were collected from IISR Shakthi and Subhakara at different time points (0.5, 1, 2 4, 8, 16, 24, 48 and 72 hai (hours after inoculation), pooled, flash frozen in liquid nitrogen and kept in −80 °C. Total RNA was extracted from both IISR Shakthi and Subhakara from un inoculated as well as challenge inoculated leaves using Trizol reagent (Invitrogen), treated with DNase I (Fermentas) to avoid genomic DNA contamination. One microgram of each RNA samples were reverse transcribed to cDNA using oligo dt (18) primers (Fermentas). The purity and concentration of the total RNA was analyzed by denaturing gel electrophoresis and spectrophotometer.

Transcriptome data of resistant variety IISR Shakthi developed using Illumina platform annotated with Blast 2Go [7] was used to retrieve the sequences. The transcript sequences of β 1, 3 glucanase and 5 R gene loci viz., Pn CNBS1, 26441, 4753, 6113 & 81were taken from transcriptome towards elucidating its function in resistance (Table 1). The locus name is hereafter denoted as Pn CNBS1, 2, 3, 4 & 5 respectively in this study. qRT PCR primers were designed with parameters: 150–200 bp maximum length, Optimal Tm at 60 °C, GC% between 45 and 65% using primer 3plus software (Table 2).

The qRT-PCR expression analysis was performed using SYBR green in 72 well plate Rotor Gene Q real time PCR systems (Qiagen, Germany). The reaction conditions were: 94 °C for 5min, 35 cycles of 94 °C for 30 s and 60 °C for 30 s. Expressions of all the six reference genes were monitored at 0.5, 1, 2, 4, 8, 12, 16, 24, 48 and 72 hai respectively. For each reaction 100 ng cDNA, 1 μM of each primer and 10 μl of SYBR green master mix (Qiagen) were added to make final volume of 20 μl per sample. Three technical replicates for the qRT-PCR were performed for the pooled biological replicates. The Pn GAPDH was used as internal control [18].

3. Results & discussion

The relative expression of PnBGlu was found to be up regulated in resistant variety (IISR Shakthi) from 0.5 h with the peak expression at 72 h while susceptible variety (Subhakara) recorded down regulation. Constitutive expression was observed in both resistant and susceptible varieties of black pepper (Fig. 1).

β Glucanase expression is only inducible [3]. Our result implicates that the expression of β 1, 3 glucanase in black pepper is inducible upon *Phyophthroa* infection and the accumulation was at greater extent in IISR Shakthi attributes for its resistance to *P. capsici*. Induced expression of glucanase by *P. capsici* in black pepper only after infection by *P. capsici* was evidenced [11] by western blot analysis. The glucanase expression was absent in the healthy or uninfected plants.

Table 2
qRT PCR Primer Sequences.

Gene Name	Primer sequence
PnCNBS1	AGGCATCTTTCCATCACAGC
	GGAGAAGTGTGAGGCTTTCG
PnCNBS2	GGACCAGCTATCCTGATCCA
	GTGGATGAAAGCATGTGGTG
PnCNBS3	TGGCACATGGATTCTGGTAA
	TTCATGGGCATCAATCTCAA
PnCNBS4	AAATCCCAACGATCAAGCAG
	ATTCTTGGATTGCCTTGTGG
PnCNBS5	ATCGGTACGCCAACAGAAAC
	TACCACCCGAATCTGCTTTC
PnGAPDH	ATGAAGGATTGGCGAGGTGG
	AGGCCATTCCAGTGAGCTTC

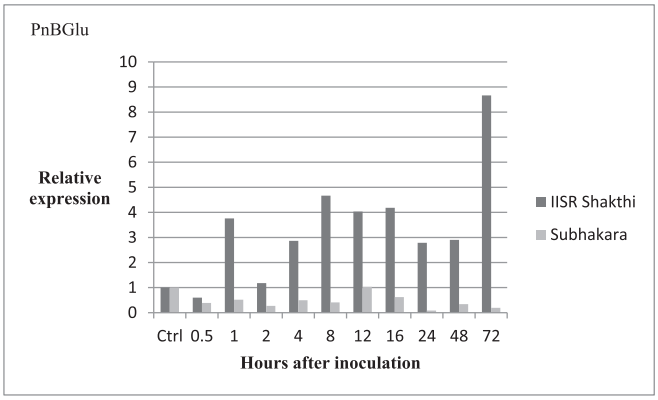

Fig 1. Expression dynamics of β 1,3 glucanase of *Piper nigrum.*

Jebakumar et al. [6] reported the presence of this enzyme from 48hpi during infection by *P. capsici* in resistant variety IISR Shakthi but the susceptible varieties (Subhakara & Panniyur 1) failed to get the reaction using western blotting. Our result is in accordance with this study with the expression of glucanase only in resistant variety. The β 1, 3 glucanase gene expression in *Phytophthora - Q. suber* pathosystem in root tissues contrary to expectations, recorded as non significance [4]. Transgenic tobacco over expressing β −1, 3-glucanase was resistant to *P. parasitica* var. *nicotianae* [19]. According to Zhang et al. [20] the expression of Ca BGlu was higher in leaves than in roots in capsicum infected with *P. capsici*. Initially the expression was down regulated and after 12hpi it increased with maximum expression at 72hpi. The expression level was higher in resistant, moderately resistant varieties compared to susceptible.

This is the first study of its kind aimed at unraveling the regulation of R genes in black pepper. The analysis of 5 R gene loci revealed the presence of NB-ARC and coiled coil domains. The PnCNBS5 and PnCNBS1 were found to have high similarity to RPM 1 type R gene. The loci PnCNBS4 PnCNBS2 & PnCNBS3 were found to have similarity to RPP13. The Blast P was used to establish the identity to the R gene loci as the comparison at the amino acid level

Table 1
BLAST identity of PnCNBS loci.

Gene	Piper TrDB locus	Assembled contig length (bp)	Top BLAST hit
PN_CNBS1	Locus_21935	1230	disease resistance protein RPM1-like [*Vitis vinifera*] 39% identity
PN_CNBS2	Locus_26441	2228	disease resistance RPP13-like protein 4 [*Malus domestica*] 39% identity
PN_CNBS3	Locus_4753	2673	putative disease resistance RPP13-like protein 1 [*Malus domestica*] 38% identity
PN_CNBS4	Locus_6113	2958	putative disease resistance RPP13-like protein 1 [Malus domestica] 28% identity
PN_CNBS5	Locus_81	1756	disease resistance protein RPM1-like [Vitis vinifera] 37% identity

is more reliable than at nucleotide level. The cluster analysis of the Piper R gene loci identified the group on similarity basis (Supplementary file 1). The phylogeny based on neighborhood using percentage identity of the deduced amino acid sequences of the Piper R gene loci identified their relatedness as two groups with each other and with the known R genes. Pn CNBS5 & PnCNBS2 were falling into one clade while the other R gene loci were falling into another clade (Fig. 2).

The conserved domain search showed that the Piper R gene Locus PnCNBS5 & PnCNBS2 with RX-CC_ like super family and NB-ARC super family domains. The Pn CNBS3 showed LRR (LRR 8 super family) and PLN00113 (Leucine rich repeat receptor −like protein kinase) domains. The Pn CNBS4 was having NB-ARC super family domain (Supplementary file 2).

In general all the 5 R gene loci showed constitutive expression. The expression dynamics varied between the resistant and susceptible genotype. Pn CNBS4 showed up regulation started at 4 hai and peaked at 12 hai as 3 fold in the resistant genotype while the susceptible recorded the down regulation in all hai (Fig. 3d). Pn CNBS2 showed the steady up regulation from 0.5 hai and peak expression as 19 fold at 8hai in susceptible where as the resistant genotype showed slight up regulation at 1hai & then constant down regulation till 72 hai (Fig. 3b). Pn CNBS5 showed early up - regulation to 3 fold at 0.5 & 4hai in resistant variety while the susceptible showed up regulation at 2hai. At 8 hai 8 fold peak expression was recorded (Fig. 3c). Pn CNBS1 recorded up regulation in resistant variety at 0.5 hai with peak expression at 8 hai as 2.7 fold increase while in susceptible only 0.5 & 8 hai showed slight up regulation otherwise all hai recorded down regulation (Fig. 3a). Pn CNBS3 showed unique pattern of expression in susceptible variety with up regulation form 0.5 hai with 10 fold increase and peaked at 8hai with 37 fold (Fig. 3c). The up regulation pattern persisted till

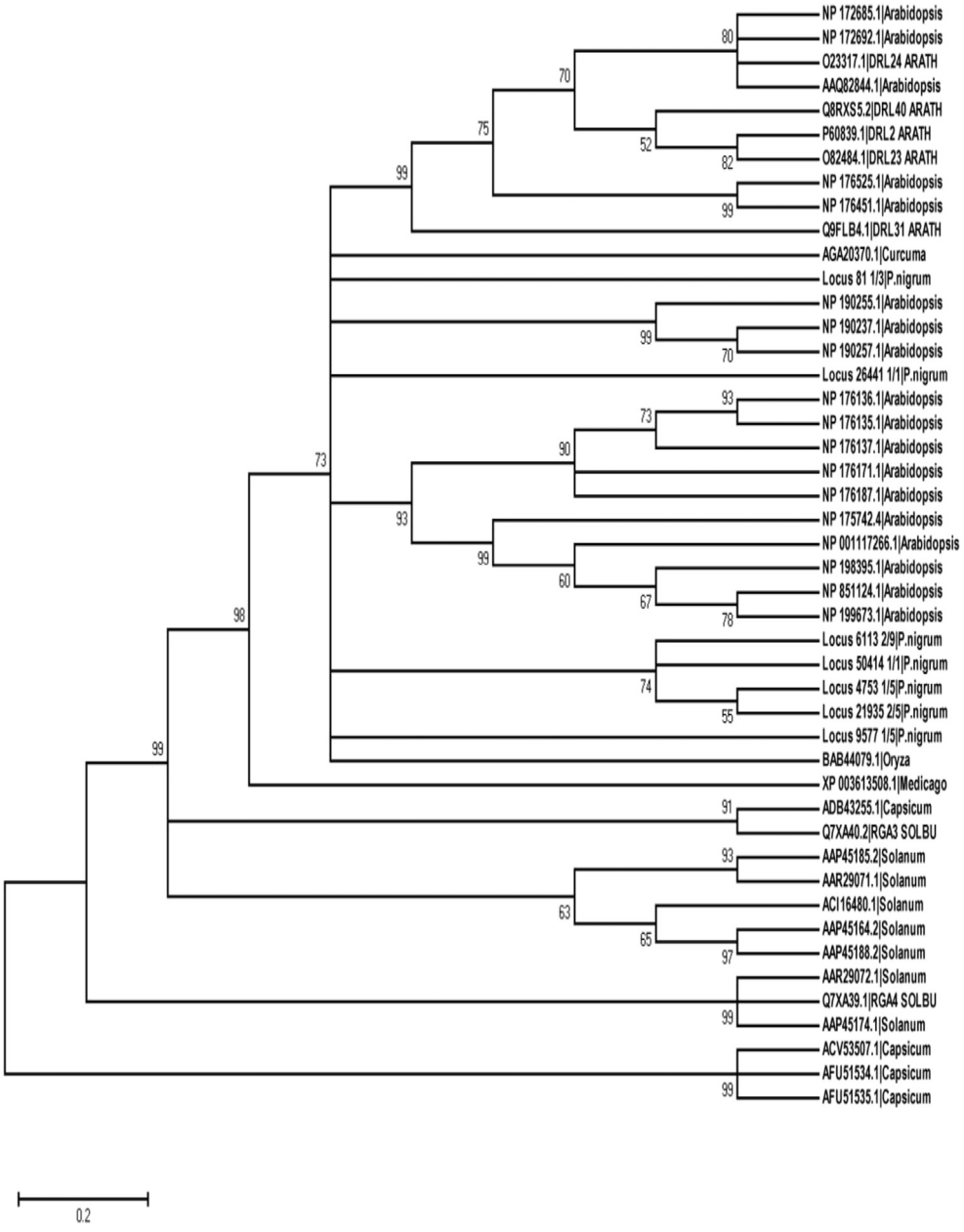

Fig. 2. *Piper nigrum* R gene Phylogeny.

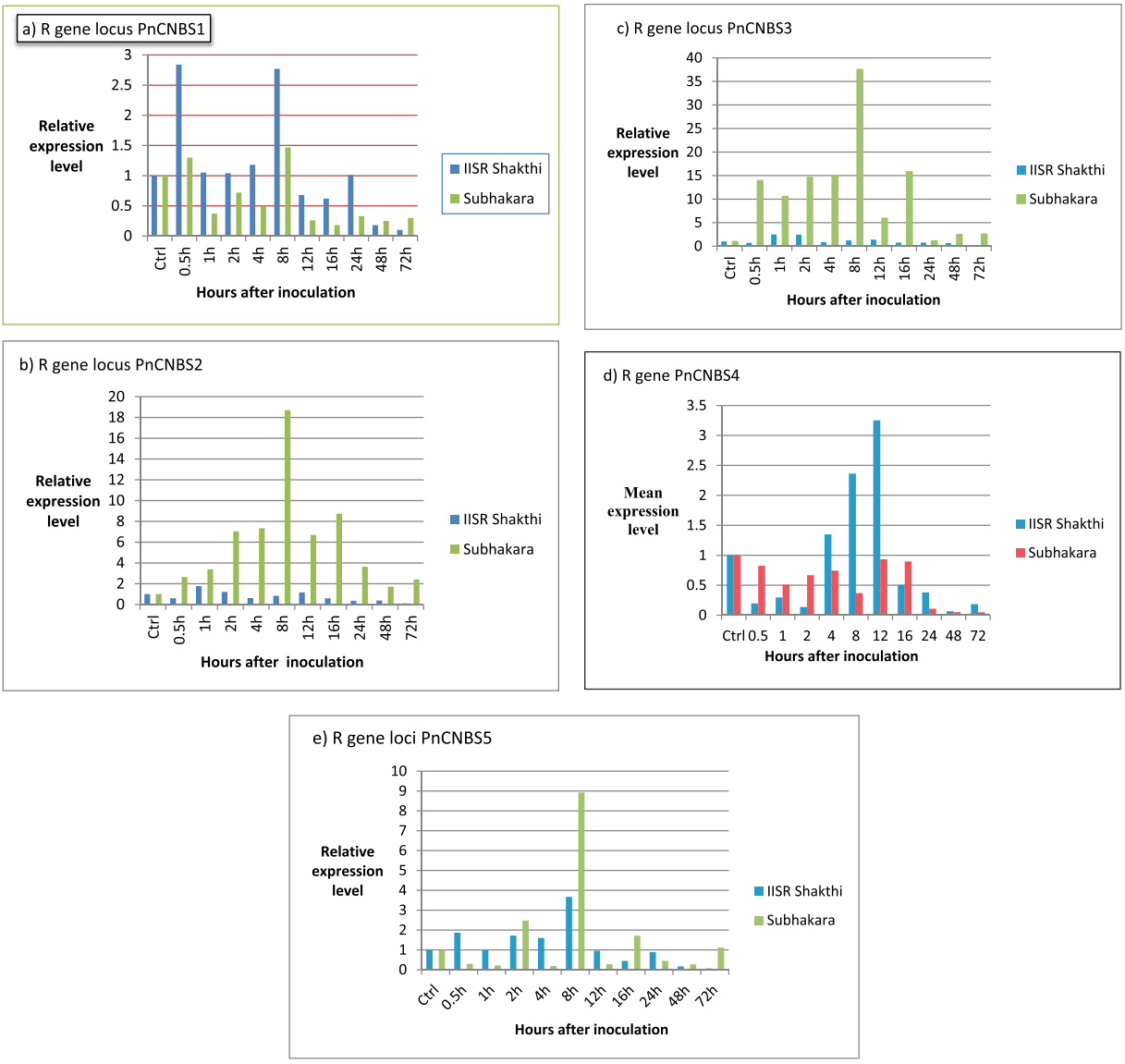

Fig. 3. a, b, c, d &e (Expression dynamics of R gene loci (PnCNBS1, 2, 3, 4, & 5 respectively) in Resistant (IISR Shakthi) and Susceptible (Subhakara) genotype.

the 72 hai when compared to resistant variety. Some members of CC-NBS-LRR gene family can be down-regulated. This has been demonstrated in the interaction *Q. suber-Pisolithus tinctorius* and *Q. robur- Microsphaera alphitoides* [9,15]. The 7.5-fold expression increase of a CC- NBS-LRR gene at 24 hpi when compared with the control from root tissue was reported *P. cinnamomi* during infection in *Q. suber* against the pathogen attack [4].

Genotype-specific disease resistance had already been demonstrated [17]. *CaRGA2* encoding a blight resistance protein showed higher level of up regulation in leaves than roots of three different pepper cultivars infected with *P. capsici*. The expression at higher levels in the resistant cultivar than in the susceptible cultivar suggests that the differences in resistance among the pepper genotypes is based on the differences in the timing and magnitude of the CaRGA2 [20]. Sliwka et al. [16] demonstrated that there were no significant changes in Rpi-phu1 transcript level in all the tested tetraploid potato lines. On the other hand, Rpi-phu1 transcription in diploid lines was inceased with *P. infestans* infection.

Quantitative real-time (qRT)-PCR analysis based correlation between transcript abundance of the *RB* gene and the level of the *RB*-mediated late blight resistance was reported by Kramer et al. [8]. High level of resistance was found associated with a combination of rapid *RB* transcript induction immediately after pathogen infection followed by the steady production of *RB* transcript in transgenic lines of potato. The quantitative analysis of resistance gene RB in transgenic potato was demonstrated to be transcribed at all ages of plant development and temperatures in all genetic backgrounds viz., Dark Red, Norland, Katahdin, and Russet Burbank [5,10]. R gene loci PnCNBS5, PnCNBS3 and PnCNBS2 showed higher expression in susceptible variety in the early hours of infection while resistant genotype recorded its down regulation during the infection till late hours suggesting that the selective down expression of these loci may be attributing resistance to the resistant variety IISR- Shakthi. These R genes may be the susceptibility genes (S-genes) in black pepper for *Phytophthora*. The R gene locus PnCNBS4 and PnCNBS1 showed high relative expression in resistant genotype at early hours (4 hai & 0.5 hai respectively) showing their direct involvement in resistance towards *P. capsici.* The differential expression of the R gene loci leads to the inference that their selection under selective pressure with the functional

significance under *P. nigrum -P. capsici* interaction.

The results presented in this study is the first of its kind study in black pepper- *Phytophthora* pathosystem and of great importance in the step towards elucidation of host defense in black pepper. It will be a valuable source to develop closely linked molecular markers that can be used in marker-assisted selection and also in recent gene editing platform towards developing resistant varieties for *Phytophthora* foot rot in this export oriented spice crop in future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pmpp.2017.10.011.

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